1903-Plat

Single Molecule Enzyme Catalysis: Steps towards Accurate Kinetic Schemes

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Single molecule fluorescence spectroscopy provides the unique possibility to study enzymatic reactions with single turnover resolution. using fluorogenic enzyme substrates, the time sequence of enzymatic turnovers can be followed and fluctuations in the turnover rate can be detected. To investigate the possibility of constructing kinetic schemes, we have studied the pH-dependent activity of the enzyme alpha-chymotrypsin.

An accurate construction of kinetic schemes is so far limited, however, by the design of most fluorogenic substrates that carry two enzyme-cleavable bonds per fluorophore. using a typical double-substituted Rhodamine 110-based substrate, the reaction proceeds in two steps involving a mono-substituted intermediate that possesses only 30% of the quantum yield of Rhodamine 110. Despite its low brightness, we are able to specifically detect the intermediate based on its different fluorescence lifetime. At high substrate concentrations the only product formed is the intermediate and a simplified but accurate kinetic scheme can be obtained. Our results show pH-dependent activity of alpha-chymotrypsin but clearly do NOT support stretched exponential kinetics, frequently interpreted as dynamic disorder. We hypothesize that ''stretched exponentials'' might be data analysis artefacts occurring for low signal-to-noise data.

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Direct Measurement of Initiation, Elongation, Splicing and Termination Kinetics in Living Human Cells

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Eukaryotic transcription involves the coordination of many multi-subunit complexes, including the pre-initiation complex, the polymerase, the spliceosome and elongation and termination factors. Most knowledge in the field is inferred from ensemble and/or in vitro assays, giving a detailed but static picture. How these macromolecular machines coordinate in vivo remains unknown. Recently, we were able to observe transcription in live yeast cells by monitoring fluorescently-tagged nascent transcripts [Larson et al. 2011, Science, 322:475]. Here, we extend this method to a dual-color system in human cells, allowing one for the first time to resolve the kinetics of initiation, elongation, splicing and termination at the same gene in a living human cell. using the orthogonal RNA-binding MS2 and PP7 bacteriophage coat proteins, we fluorescently labeled the largest intron and the 3'UTR of a stably integrated reporter gene. Fluorescence fluctuations recorded simultaneously in both channels at the transcription site reflect the initiation of pre-mRNA synthesis, the elongation kinetics of the polymerase, and the kinetics of intron and transcript release. We developed an approach based on cross-correlation to reveal the relative timing of these events for single transcripts. To that end, we derived a mathematical model that predicts the correlation functions depending on the timing of the underlying processes, allowing us to test hypotheses about polymerase progression and pausing at the single-molecule level in vivo. We observe an elongation speed of 1.5 kb/min, which is measured independently of initiation and termination. No pausing was detected in the body of the gene, but termination took an average of ~200 sec, during which co-transcriptional splicing was observed for a fraction of transcripts. These results raise the possibility that co-transcriptional RNA processing may result in a kinetic checkpoint at termination rather than pauses during elongation.

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Total Internal Reflection Fluorescence Microscopy Imaging-Guided Confocal Single-Molecule Fluorescence Spectroscopy

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We have demonstrated a new integrated single-molecule spectroscopy approach by using total internal reflection fluorescence microscopy imaging to guide confocal single molecule fluorescence spectroscopic measurements. With the localization by two-dimensional Gaussian fit and coordinates transformation, any single-molecule fluorescence spot of interest in the imaging field of TIRFM imaging mode is accurately registrated and shifted to the excitation focus of confocal mode for single-molecule fluorescence spectroscopic measurements, through a computer-controlled close loop piezoelectric x-y scanning stage. Our integrated system have a number of significant advantages including the low requirement for laser power of the femtosecond pulse laser, avoiding of photon bleaching by wide field excitation, high signal collection efficiency for the confocal single-molecule fluorescence spectroscopic measurements. We have demonstrated the capability of both multiple molecules sampling simultaneously and in-situ fluorescence time-resolved analyses for pin-point individual molecules. It is applicable to conduct the measurements of the spatially and temporally stochastic single-molecule on interfaces. We have applied this technical approach to study single-molecule enzymatic reaction product releasing dynamics.

Platform: Membrane Dynamics & Bilayer Probes

1906-Plat

Physiological Calcium Concentrations Induce PI(4,5)P2 Clustering: PI(4,5)P2 as a Lipidic Calcium Sensor

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Phosphatidylinositol 4,5-bisphosphate (PI(4,5)P2) is a minor component of the plasma membrane of eukaryotic cells that is essential for several cellular mechanisms, including organization and remodeling of the actin cytoskeleton, membrane trafficking, endocytosis and exocytosis. The regulation of these processes is thought to involve localized enrichment of PI(4,5)P2 in the plasma membrane at particular timings. Particularly in calcium-triggered SNARE-dependent exocytosis, PI(4,5)P2 is known to interact and co-segregate with certain synaptic proteins. However, the mechanisms responsible for PI(4,5)P2 clustering in particular sites of the plasma membrane are still not completely understood.

Using membrane model systems and fluorescently labeled PI(4,5)P2, we were able to demonstrate that PI(4,5)P2 lateral distribution is sensitive to physiologic calcium concentrations, in the low micromolar range. Calcium concentrations of 0-100 μM were used to mimic neuron intracellular calcium concentrations before and after a stimulus. Fluorescence self-quenching and energy migration data obtained from steady-state and time-resolved fluorescence methods clearly showed that Ca^{2+} ions promoted the clustering of PI(4,5)P2 molecules in both liquid disordered and liquid ordered membranes. Sensitivity of PI(4,5)P2 clustering to [Ca^{2+}] is shown to be highly dependent on both PI(4,5)P2 density and membrane lipid phase.

In this way, the lateral distribution of PI(4,5)P2 presents significant sensitivity to changes in calcium concentration of only a few micromolar, allowing this lipid to function as a lipidic calcium sensor. In the particular case of SNARE-dependent exocytosis, it is expected that localized increases in calcium concentration will dramatically change the pattern of PI(4,5)P2 interaction with synaptic proteins, possibly enhancing fusion rates and promoting exocytosis.

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1907-Plat

Targeting Cellular Organelles by Amphipathic Helices: Taking Advantage of Membrane Biophysics

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Eukaryotic cells are characterized by a rich variety of membranes, that differ by both morphology and lipid composition. These membranes form the boundaries of the different cellular organelles, that can be selectively targeted by certain peripheral proteins to trigger specific signaling and trafficking pathways.

Using atomistic molecular dynamics simulations and new analysis tools we have recently been able to understand at the molecular level how a family of membrane curvature sensors, the Amphipathic Lipid Packing Sensor (ALPS) motifs, can specifically bind to lipid bilayers that are abundant in lipid packing defects, such as those induced by membrane curvature.

Since organelle membranes are identified by their characteristic lipid composition and geometrical shape, we resort to a coarse grained approach based on the MARTINI force field to quantify in a systematical way the effect of these two parameters on the statistical distribution of lipid packing defects in model membranes. This allows us to identify how the peculiar amino acid composition of different amphipathic helices modulates their preferential interaction with specific membranes by taking advantage of their unique physicochemical features.